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(54) [Title of the Invention] MAMMALIAN EMBRYONIC STEM CELLS, METHOD OF ESTABLISHING THE SAME, AND METHOD OF SUBCULTURING THE SAME

(57) [Abstract]
[Problems to Be Solved by the Invention] Provided are embryonic

stem cells having pluripotency, a method of establishing the same, and a method of subculturing the embryonic stem cells.
[Means of Solving the Problems] Mammalian embryonic stem cells having all of the following cell biological features: (1) derived from an embryo in the blastocyste stage, (2) continue to grow in the undifferentiated state, (3) express the sugar chain SSEA-1 antigen, (4) positive for alkaline phosphatase activity, (5) express the transcription factor Oct3/4, and (6) have pluripotency; a method of establishing embryonic stem cells comprising culturing cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα containing fetal calf serum as the medium to form colonies; and a method of subculturing embryonic stem cells.

[Claims]
[Claim 1] Mammalian embryonic stem cells having all of the following cell biological features: (1) derived from an embryo in the blastocyste stage, (2) continue to grow in the undifferentiated state, (3) express the sugar chain SSEA-1 antigen, (4) positive for alkaline phosphatase activity, (5) express the transcription factor Oct3/4, and (6) have pluripotency.
[Claim 2] The embryonic stem cells according to claim 1, wherein the mammal is a rat, a bovine or a horse.
[Claim 3] A method of establishing embryonic stem cells, comprising culturing cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα containing fetal calf serum as the medium to form colonies.
[Claim 4] A method of establishing embryonic stem cells, comprising culturing cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα containing fetal calf serum as the medium to form

colonies, and screening these colonies with growth potential in the undifferentiated state, the expressibility of the sugar chain SSEA-1 antigen, the presence or absence of alkaline phosphatase activity, the expressibility of Oct3/4, and the presence or absence of pluripotency as indexes.

[Claim 5] The method of establishing embryonic stem cells according to claim 3 or 4, comprising culturing cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage with an MEMα medium containing fetal calf serum, epithelial growth factor, and leukemia inhibitory factor.

[Claim 6] The method of establishing embryonic stem cells according to one of claims 3 to 5, wherein the mammal is a rat, a bovine or a horse.

[Claim 7] A method of subculturing embryonic stem cells, comprising subjecting cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage to primary culture on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα containing fetal calf serum as the medium to form colonies, detaching the cells of the colonies from the medium using a trypsin EDTA solution, applying the detached cells, along with a PBS solution, to a centrifuge to wash the cells and disperse them into discrete cells, and thereafter re-culturing the cells with the medium used for the above-described primary culture.

[Claim 8] The method of subculturing embryonic stem cells according to claim 7, comprising subjecting cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage to primary culture with an MEMα medium containing fetal calf serum, epithelial growth factor, and leukemia inhibitory factor.

[Claim 9] The method of subculturing embryonic stem cells according to claim 7 or 8, wherein the mammal is a rat, a bovine or a horse.

[Detailed Description of the Invention]

[0001]

[Field of the Invention] The present invention relates to

embryonic stem cells, a method of establishing the embryonic stem cells, and a method of subculturing the embryonic stem cells. More specifically, the present invention relates to embryonic stem cells that are valuable as a research material for elucidating the cell biological and molecular biological control converting function for differentiation from embryonic stem cells to organs, and from organs to individuals, or as a medical material for creating organs for heterologous transplantation, and as donor cells for producing recombinant clone animals that serve as bioreactors for useful pharmaceuticals because of the possibility that embryonic stem cells are likely to undergo homologous recombination with the extraneous gene at high frequency, a method of establishing the embryonic stem cells, and a method of subculturing the embryonic stem cells.

[0002]

[Prior Art] Since the discovery of mouse embryonic stem cells as cultured cells capable of differentiating into individual organisms, embryology aiming at tissue/organ regeneration, elucidation of mechanisms for embryogenesis, and creation of clone individuals using the embryonic stem cells has been in the limelight, and is expected as an approach to which new-generation medicine and biotechnology is directed.

[0003] Since the first report of the establishment of embryonic stem cells in humans by a group of US researchers in 1998 (Thompson TA, et al., Science 282, 1145-1147, 1998; Shamblott MJ, et al., Proc. Natl. Acad. Sci. USA. 1995, 13726-13731, 1998), embryonic stem cells have been highlighted as versatile cells that will open the way to regenerative medicine for the human nervous systems, blood cell systems, organs and the like, and to gene therapy. However, none has succeeded in producing a germline chimera using embryonic stem cells (or embryonal stem-like cells) in any animal species other than the mouse (SticeS, et al., Biol. Reprod. 48, 715-719, 1996; Strelchenko N, Therio-genology, 37, 111-126, 1996); the mouse remains the only animal species in which recombinant individuals can be produced

via transformed stem cells obtained by gene manipulation of embryonic stem cells ex vivo (Bradley A, et al., Nature, 399, 255, 1984).

[0004] Strelchenko reported in the aforementioned paper that when bovine embryonal stem-like cells were established, nuclear transplantation was performed with these cells as the donor nucleus, and a clone embryo was transplanted to a recipient cow, the cow suffered abortion on day 55 of gestation. However, the established cells tested negative for alkaline phosphatase expression, a feature of stem cells mentioned by the present inventors, and it seems that the established cells did not clear other requirements. Although Campbell et al. succeeded in creating clone offspring from cultured cells derived from embryonic discs in sheep (Nature, 380, 64-66, 1996), they did not describe the expressibility of alkaline phosphatase or the expressibility of the sugar chain SSEA-1 antibody, which are features of embryonic stem cells, and the cells derived from embryonic discs seem to be morphologically epithelium-like cells.

[0005] Strelchenko, Campbell and others used primary culture fibroblasts derived from mouse STO, which is a fetal fibroblast line, or from a mouse fetus, to culture embryonal stem-like cells. Primary culture fibroblasts, which are used to maintain embryonic stem cells, are reported not to permit passage culture when used for primary culture, although a cell line can be used when they are used after secondary culture of primordial germ cells (Stewart, CL, et al., Dev. Biol. 161, 626-628, 1994); it is considered to be likely that in establishing embryonic stem cells of domestic animals such as bovines and sheep, the establishment of stem cells was hampered by the use of primary culture fibroblasts thereof. Embryonic stem cells are also known to maintain the undifferentiated state and exhibit promoted cell growth in the presence of leukemia inhibitory factor. However, even with the addition of leukemia inhibitory factor, none has succeeded in establishing embryonic stem cells in rodents other than the mouse or

domestic animals; an unknown growth factor may exist.
[0006] As stated above, no animal species, other than the mouse and the human, have been found to have established embryonic stem cells, and there are no reports demonstrating the expression of the sugar chain SSEA-1, which is essential for identifying embryonic stem cells, the manifestation of pluripotency in ex vitro culture systems and the like.
[0007]
[Problems to Be Solved by the Invention] The present invention has been developed in view of the above-described circumstances, and is intended to provide embryonic stem cells other than those of the mouse and human. The present invention is also intended to provide a method for establishing such cells and a method for subculturing such cells.
[0008]
[Means for Solving the Problems] To solve the above-described problems, a first embodiment of the present invention provides mammalian embryonic stem cells having all of the following cell biological features: (1) derived from an embryo in the blastocyst stage, (2) continue to grow in the undifferentiated state, (3) express the sugar chain SSEA-1 antigen, (4) positive for alkaline phosphatase activity, (5) express the transcription factor Oct3/4, and (6) have pluripotency.
[0009] The present invention also provides a method of establishing embryonic stem cells, comprising culturing cells obtained from an inner cell mass of a mammalian embryo on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα comprising fetal calf serum as the medium to form colonies, or screening these colonies with the presence or absence of alkaline phosphatase activity, the expressibility of the sugar chain SSEA-1 antigen, the expressibility of Oct3/4, the presence or absence of pluripotency, and growth potential in the undifferentiated state as indexes.
[0010] Furthermore, the present invention provides a method of subculturing embryonic stem cells, comprising subjecting cells obtained from an inner cell mass of a mammalian embryo in the

embryonic stem cell stage to primary culture on umbilical endothelial cells isolated from the umbilical cord as feeders with an MEMα containing fetal calf serum as the medium to form colonies, detaching the cells of the colonies from the medium using a 0.25% trypsin 0.1% EDTA solution, applying the detached cells, along with a PBS solution, to a centrifuge to wash the cells, and disperse them into discrete cells, and thereafter re-culturing the cells with the medium used for the above-described primary culture.

[0011] In the above-described method of establishing embryonic stem cells and the method of subculturing the same, it is a preferred embodiment wherein the cells obtained from an inner cell mass of a mammalian embryo in the blastocyst stage are cultured with an MEMα medium comprising fetal calf serum, epithelial growth factor, and leukemia inhibitory factor.

[0012]

[Mode for Carrying out the Invention] First, the method of establishing embryonic stem cells of the present invention is described in more detail. Usually, for establishing embryonic stem cells, mouse STO feeder cells wherein cell division is terminated by mitomycin treatment or irradiation with γ radiation are needed. In the present invention, umbilical endothelial cells are used as feeders. The culture medium is supplemented with fetal calf serum (FCS) and leukemia inhibitory factor (LIF). Although epithelial growth factor (EGF) is not essential for the formation of embryonic stem cell colonies, it has a remarkable effect in establishing embryonic stem cells; it is important that EGF be added as a growth factor for the cells to the culture medium. Using umbilical cord cells as feeders and adding EGF to the culture medium synergistically act on the growth of embryonic stem cells and enable the establishment of a cell line.

[0013] The amounts of these ingredients added to the medium can be, for example, 5 to 10% for FCS, 10 to 50 ng/ml for LIF, and about 10 to 50 ng/ml for EGF. Cultivation is performed in the presence of 5% CO_2 at a temperature of around 39°C. By the

cultivation described above, colonies of embryonic stem cells are obtained. Furthermore, by screening cells that form these colonies with, for example, expression of the sugar chain SSEA-1 antigen, positivity for alkaline phosphatase, expression of the transcription factor Oct3/4, and pluripotency, as indexes, functional expression as embryonic stem cells can be confirmed.

[0014] For example, the expressibility of the SSEA-1 antigen can be confirmed by staining the cells with the SSEA-1 antibody, and then separating the cells by flowcytometry. Pluripotency can be confirmed by differentiating the cells into nerve cells using a culture system, and staining the cells with the glial fibrillary acidic protein (GFAP) or Nestin antibody marker. Furthermore, pluripotency can also be confirmed by transferring the nucleus of an embryonic stem cell to a denucleated unfertilized egg derived from ex vivo culture, culturing the egg ex vivo to allow the egg to develop into a blastocyst, and transferring this blastocyst to the uterus of an embryo recipient female to make the female to be pregnant.

[0015] Described below is the method of subculturing embryonic stem cells according to the present invention. This method comprises detaching colonies of embryonic stem cells obtained using the above-described establishment method (primary culture cells) from the incubator, and re-culturing the cells in another incubator to allow them to grow. The colonies, along with the feeder cells, can be detached by removing the culture medium from the incubator, threafter adding an about 0.1 to 0.25% trypsin EDTA solution, and treating the colonies at about 39°C for about 6 minutes. By culturing the colonies with the same medium as that used for the primary culture of cells derived from umbilical endothelial cells wherein cell division has been terminated (i.e., a medium containing FCS, LIF, and EGF), the colonies can be allowed to adhere to the feeders, and to grow again. Except the choice of cells used for the subculture, the colonies can also be stored under freezing in liquid nitrogen by a conventional method.

[0016] As stated above, the method of the present invention is

applicable to the establishment of embryonic stem cells of all mammals, including humans, and enables the establishment of embryonic stem cells from a variety of animal species, and the subculturing of the embryonic stem cells. For example, swine, bovine and equine embryonic stem cells can be utilized to prepare organs for heterologous transplantation, can also be utilized to produce recombinant clone animals that serve as bioreactors for useful pharmaceuticals, and are expected to make significant contributions to advances in regenerative medicine and biotechnology.

[0017] The method of establishing stem cells of the present invention is hereinafter described in more detail and more specifically by means of the following Examples, and the features of the embryonic stem cells obtained using this method are described by reference to test results. However, the present invention is not by any means limited by the Examples below.

[0018]

[Examples] Example 1: The embryonic stem cells of the present invention were acquired by the method described below, and the cells were tested for biological characteristics.

[0019] (1) Described below are how to separate and culture umbilical endothelial cells, and preparatory work for their use as feeder cells. The umbilical cord of a neonatal bovine was cut into about 1 cm^2 pieces using sterile surgical scissors, 70% ethanol was sprayed, and flame sterilization was performed, after which the pieces were washed with a PBS (-) supplemented with antibiotics (Dulbecco, free from Ca and Mg) several times (penicillin 2000 units/ml, Meiji Seika Kaisha, Ltd.; streptomycin 100 μg/ml, Meiji Seika Kaisha, Ltd.; Fungizone 25 μg/ml, GIBCO BRL). Furthermore, the above-described sample was shredded into about 1 mm^2 pieces on a sterile Petri dish, after which the shredded pieces were treated with 3 ml of 0.25% trypsin 0.1% EDTA (GIBCO BRL) for 10 minutes. This trypsin liquid containing the sample was diluted with 5 ml of PBS (-), after which it was centrifuged (1000 rpm, 5 minutes), and the

precipitate was suspended in 5 ml of PBS (-) and again centrifuged under the same conditions. The precipitate obtained was placed in a culture dish (Primaria, Falcon, 6 cm diameter) containing 3 ml of an MEMα (GIBCO BRL) containing 10% FCS (GIBCO BRL) and antibiotics, and cultured under the conditions of 38.6°C and 5% CO_2. After 3 to 4 days of cultivation, endothelial cells appeared as colonies around the shredded umbilical cord sample. After the cells were further cultured for 6 to 10 days until confluency was reached, the umbilical cord sample mass was removed, after which subculture was performed. The medium was exchanged with a fresh supply every two days. Subculture was performed by washing the cells with PBS (-) twice, and thereafter treating them with trypsin EDTA for several minutes, and sewing the cells to a 6 cm culture dish at an about 1/5 concentration. Four days later, the cells became nearly confluent. After passage was repeated 2 to 3 times, the cells were stored under freezing by a conventional method. When used as feeders, frozen cells were thawed, after which cultivation was newly begun. Procedures for preparing the feeder cells: a) mitomycin (Sigma) was added to nearly confluent umbilical endothelial cells at 10 μg/ml, and the cells were cultured for 3 to 4 hours. b) The medium was removed, the cells were washed with PBS (-) three times and trypsinated, and the cells were detached, after which the cells were centrifuged (1000 rpm, 5 minutes). The cells were suspended to a concentration of $2x10^6$/ml. c) The suspension was added drop by drop to a 4-well culture dish (Nunc) at 0.5 ml per well. Cells at 2 days or more after preparation, which can serve as uniform feeder cells, were used to establish embryonic stem cells.

[0020] (2) Establishment and cultivation of embryonic stem cells

Embryos, previously stored under freezing, were thawed by a conventional method, after which they were tested. The embryos used were embryos in the blastocyst stage at 4 days after confirmation of copulation for rats, and embryos in the

blastocyst stage at 7 days after artificial or spontaneous mating, respectively, for bovines and horses. An inner cell mass (ICM) portion was cut out from each of all the blastocysts by microsurgery, and cultured on feeder cells. Cultivation was continued in an MEMα (10% FCS, containing penicillin and streptomycin) supplemented with 20 ng/ml of EGF (Sigma) and 20 ng/ml of LIF (Sigma) at 38.6°C in the presence of 5% CO_2. The medium was exchanged with a fresh supply every two days. The ICM on the feeders adhered to the feeders in 1 to 2 days, and continued to grow. After 4 to 5 days of cultivation, cell colonies began to develop around the ICM. Cultivation was further continued; when the colony diameter became 3000 to 4000 μm, the cells were detached from the feeders using a pipette and treated with a trypsin solution for about 6 to 7 minutes, after which the cells were dissociated using a glass pipette. After dissociation, the cells were diluted with PBS (-) and then twice centrifuged and washed, after which the precipitate obtained was sown onto feeder cells at a 1/3 concentration under the same culture conditions as those described above (primary culture).

[0021] (3) Test method

Morphology, one of the indexes of embryonic stem cells, was examined for growth in the undifferentiated state under a phase-contrast microscope. Alkaline phosphatase activity, a feature for undifferentiated cells, was detected by staining with Naphthol AS MX Phosphate (Sigma) containing fast red TR salt (Sigma). The expression of the SSEA-1 antigen, which is a marker for undifferentiated embryonic stem cells, was confirmed by attempting to separate a population of stem cells that bind to the SSEA-1 antibody by anti-SSEA-1 antibody staining using flowcytometry (fluorescence activated cell sorter; FACS). That is, after $2x10^5$ rat embryonic stem cells were reacted with the anti-rat IgG and anti-mouse SSEA-1 antibodies, they were secondarily stained with FITC (fluorecein isothio-cyanate)-labeled rabbit anti-mouse IgG and analyzed by FACS. Also, after $2x10^5$ bovine embryonic stem cells were reacted with the

anti-bovine IgG and anti-mouse SSEA-1 antibodies, they were secondarily stained with FITC-labeled rabbit anti-mouse IgG and analyzed by FACS. Furthermore, for horses as well, after $2x10^5$ embryonic stem cells were reacted with the anti-equine IgG and anti-mouse SSEA-1 antibodies, they were secondarily stained in the same manner as with rats and bovines and analyzed by FACS. Detection of the transcription factor Oct3/4, which is used to identify mouse undifferentiated cells, was performed by a PCR method. To examine the pluripotency of the embryonic stem cells, the stem cells obtained were differentiated into nervous system cells, and an attempt was made to detect astroglia and nerve stem cells cell immunochemically using the GFAP and Nestin antibodies, which are cell marker antibodies therefor. That is, the equine or rat embryonic stem cells were subcultured three times using an MEMα medium containing EGF, fibroblast growth factor (FGF)2, and FGF9; when glial cells were observed, they were reacted with the mouse anti-Nestin antibody, and thereafter they were secondarily reacted with the alkaline phosphatase conjugate anti-mouse IgG antibody and stained with Fast red. Likewise, after the cells were subcultured three times using a medium containing EGF, FGF2, and FGF9, these cytokines were removed; after the cells were further cultured for 7 days, they were reacted with the rabbit anti-GFAP antibody, and thereafter they were secondarily reacted with the alkaline phosphatase conjugate anti-rabbit IgG antibody, and stained in the same manner. Furthermore, to examine the pluripotency of the bovine embryonic stem cells, nuclear transplantation was performed with the stem cells as the nuclear donor to create a clone embryo, after which the embryo was transplanted to an embryo recipient female. Using control media deprived of one or both of EGF and LIF, which are factors added to the medium, equine embryonic stem cells were cultured over 3 to 4 generations, and the effects of the additive factors on the cell growth were examined. To examine the effect of the feeder cells on the establishment of the stem cells, bovine ICMs were cultured in the absence of the feeder

cells, and the results were compared with those from controls.

[0022] (4) Test results

As a result of cultivation under the above-described conditions (2), embryonic stem cell colonies of densely gathered cells very similar to mouse embryonic stem cells were obtained, as shown in the phase-contrast photomicrographs in Figures 1 to 3. In primary culture, it took 7 to 10 days to reach confluency. The cells tested positive for alkaline phosphatase activity (Figures 4, 5, and 6). The ratio of anti-mouse SSEA-1 antibody-positive cells to all the embryonic stem cells is shown in Figures 7, 8, and 9. The ratio was 24.5% for the rats, 10.2% for the bovines, and 38.4% for the horses, suggesting differences among the animal species.

[0023]

[Table 1]

Results of detection of Oct3/4 in the various cell lines established

cell line	Oct3/4
Rat WDA 1	+
Bovine WA 3	+
Equine EK 1	+
Bovine umbilical endothelium	—

As is evident from Table 1, the embryonic stem cells of all the test animal species tested positive for Oct3/4.

[0024] Next, as evidence for the pluripotency of the embryonic stem cells, cells positive for the GPAP antibody, which is an astroglia marker (Figure 10), and cells positive for the Nestin antibody, which is a nerve stem cell marker (Figure 11), were produced using an ex vivo culture system. From this result, it was demonstrated that the equine embryonic stem cells have pluripotency. Similar results were obtained with the rat embryonic stem cells.

[0025]

[Table 2]

Bovine embryonic stem cell clone embryo generation rates

Experiment	Number of generations	Number of embryos supplied	Number of embryos fused	Number of blastocysts	Number of animals receiving transplantation	Number of fertilized females
1	11	153	87(56.9)	11(12.6)	8	4(50.0)
2	12	110	92(83.6)	22(23.9)	10	6(60.0)
3	12	122	98(80.3)	28(28.6)	12	7(58.3)
total		385	277(71.9)	61(22.0)	30	17(56.7)

*: By ultrasonic diagnosis.

[0026] Furthermore, the pregnancy rates for nucleus-transplanted embryos with bovine embryonic stem cells as the donor nucleus are shown in Table 2. Thereby, the pluripotency of the cell nucleus was also confirmed.

[0027] As shown in Figures 1 to 11 and Tables 1 and 2 above, it was confirmed that the cell lines established in the present invention completely exhibit features as embryonic stem cells having pluripotency.

[0028] As is evident from Figure 12, the cells did not became confluent even after being cultured for 1 week in the control medium devoid of both EGF and LIF, whereas the cells became confluent after 4 days of cultivation in the system with the addition of EGF alone, and after 3 days in the system with a combination of EGF and LIF; a remarkable growth promotive effect was observed.

[0029]

[Table 4]

Effects of feeder cells derived from umbilical endothelial cells on the establishment of bovine embryonic stem cells

	Feeder cells (+)	Feeder cells (−)
Number of ICMs	9	10
Number of cell lines established (%)	5(56)	0

*: The medium used was an MEMα supplemented with EGF and LIF + 10% FCS.

[0030] As is evident from Table 4, bovine embryonic stem cells could not be established in the absence of feeder cells, but when feeder cells were used, five (56%) of the nine ICMs were established as stem cells, suggesting that using feeder cells may be essential for the establishment of embryonic stem cells.

[0031] Example 2: The embryonic stem cells obtained in Example 1 were subcultured by the method described below. After the medium

was removed from the culture dish of bovine embryonic stem cells that became confluent on the feeder cells, the bovine embryonic stem cells were treated with 0.25% trypsin 0.1% EDTA for 6 to 10 minutes to disperse the embryonic stem cells and the feeder cells in the solution. This suspension was twice centrifuged (1000 rpm, 5 minutes), and the precipitate obtained was sown to a medium consisting of the same composition as that used for the subculture at a concentration of $2x10^5$ cells/ml; the cells were found to exhibit vigorous division and growth on the culture dish (Figure 13), and became nearly confluent in 4 to 5 days.

[0032]

[Effect of the Invention] As described in detail above, the present invention provides embryonic stem cells, a method of establishing the cells, and a method of culturing the embryonic stem cells over generations. These embryonic stem cells and methods can be utilized for elucidating the mechanisms for the regeneration and differentiation of stem cells, for preparing organs for heterologous transplantation, and for producing recombinant clone animals that serve as bioreactors for useful pharmaceuticals, and are expected to contribute to advances in regeneration medicine and biotechnology.

[Brief Description of the Drawings]

[Figure 1] Photomicrograph of rat embryonic stem cell colony, x 200.

[Figure 2] Photomicrograph of bovine embryonic stem cell colony, x 200.

[Figure 3] Photomicrograph of equine embryonic stem cell colony, x 100.

[Figure 4] Photomicrograph of rat embryonic stem cell colony stained with alkaline phosphatase, x 200.

[Figure 5] Photomicrograph of bovine embryonic stem cell colony stained with alkaline phosphatase, x 200.

[Figure 6] Photomicrograph of equine embryonic stem cell colony stained with alkaline phosphatase, x 200.

[Figure 7] Results of FACS of rat embryonic stem cells with anti-SSEA-1 antibody. The solid black area indicates the ratio of positive cells.

[Figure 8] Results of FACS of bovine embryonic stem cells with anti-SSEA-1 antibody. The solid black area indicates the ratio of positive cells.

[Figure 9] Results of FACS of equine embryonic stem cells with anti-SSEA-1 antibody. The solid black area indicates the ratio of positive cells.

[Figure 10] Photomicrograph of GFAP antibody-positive cells derived from equine embryonic stem cells, x 100.

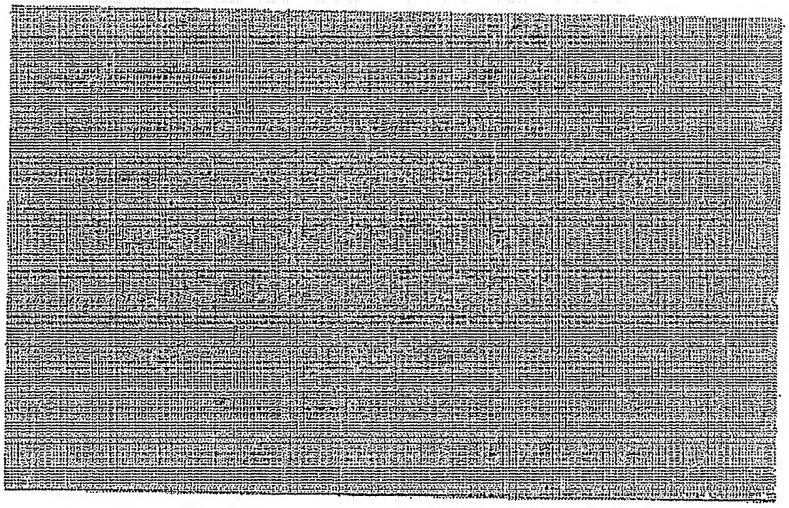
[Figure 11] Photomicrograph of Nestin antibody-positive cells derived from equine embryonic stem cells, x 200.

[Figure 12] Graph showing the effects of cell growth factor on growth of equine embryonic stem cells.

[Figure 13] Photomicrograph of bovine embryonic stem cells (5 generations), x 200.

Fig. 1

photograph substituted for drawing



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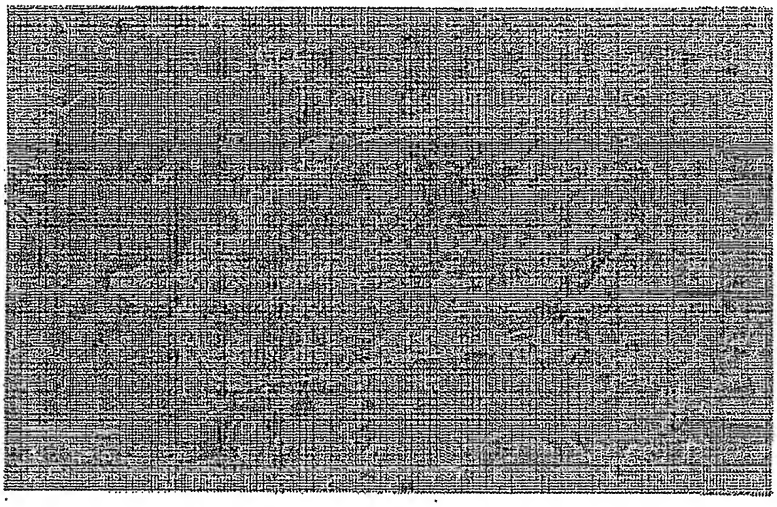

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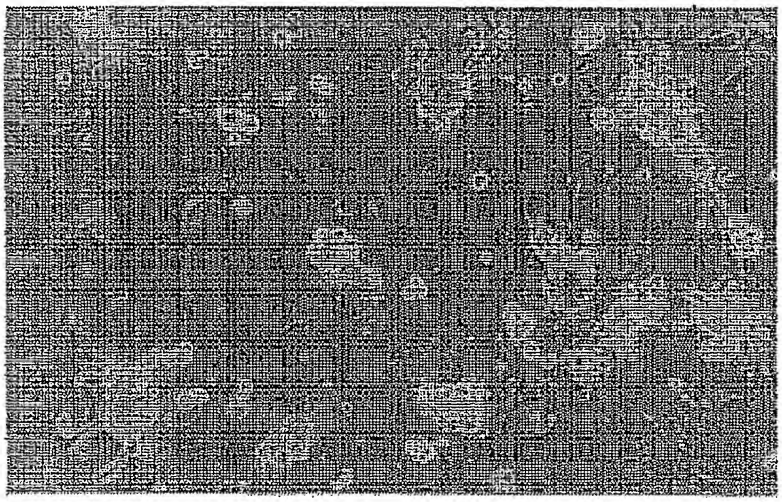

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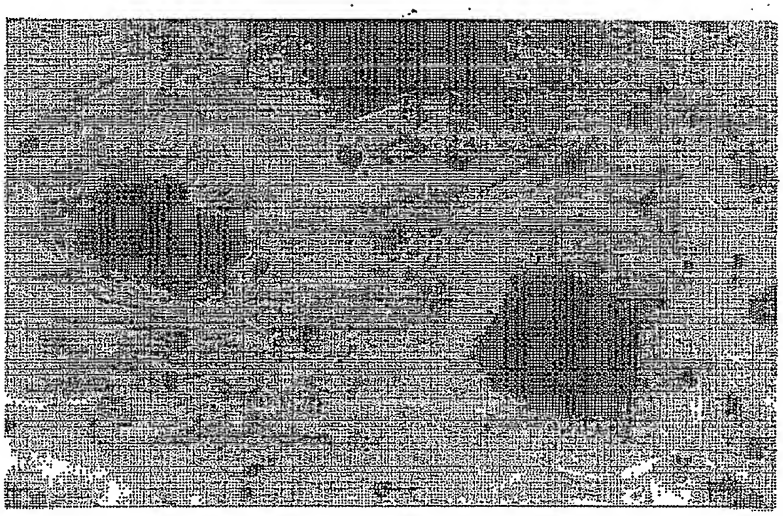

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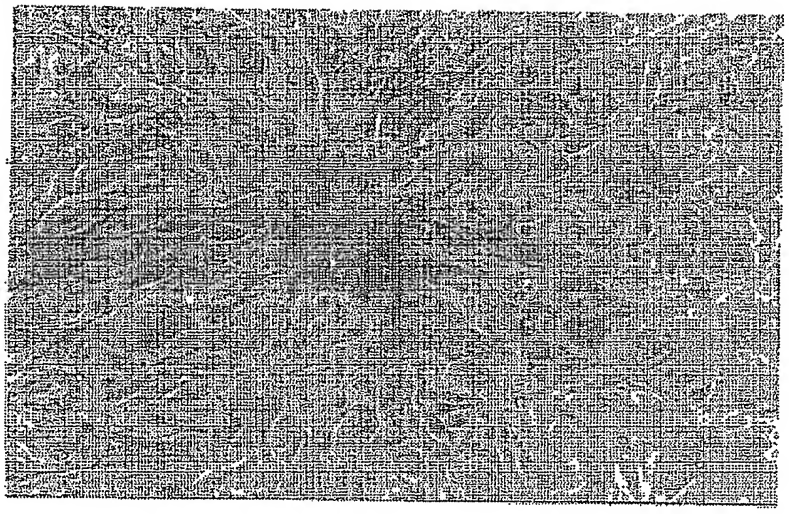

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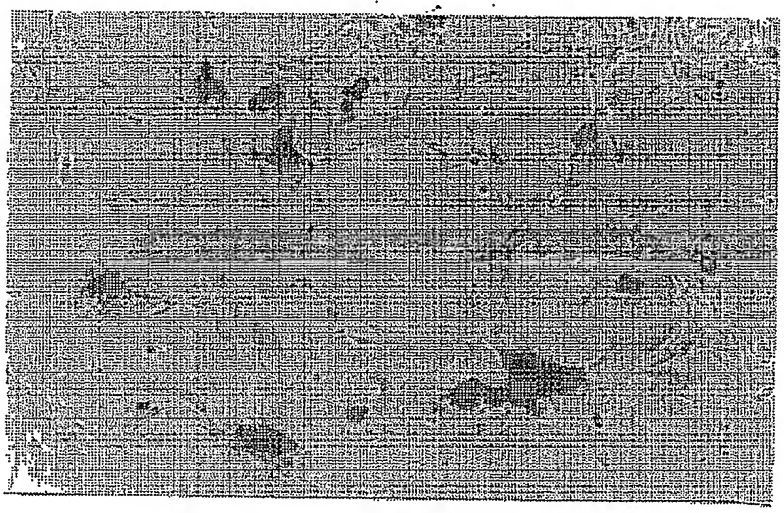

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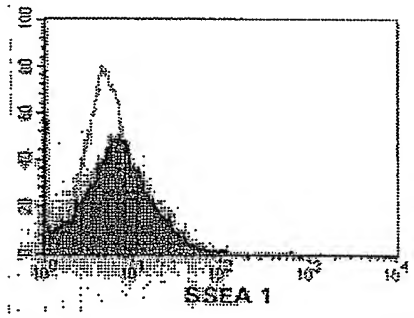

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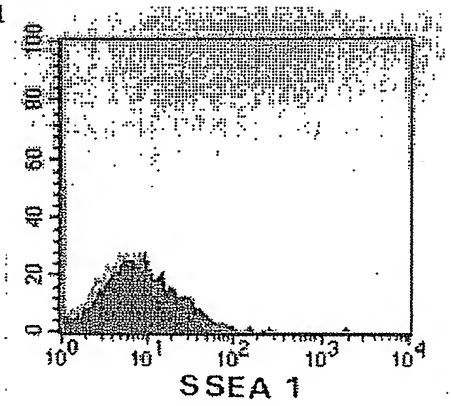

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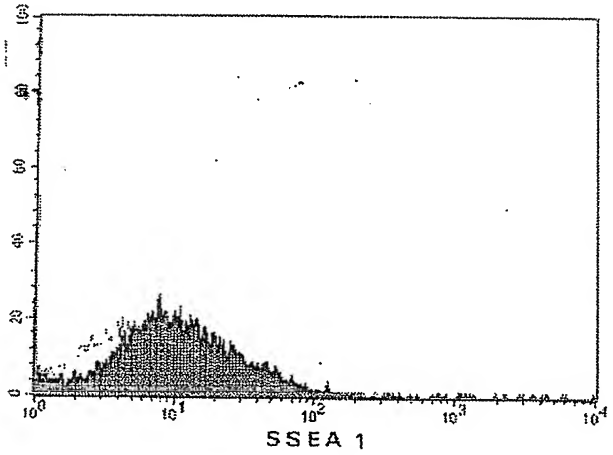

Fig. 10

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for drawing

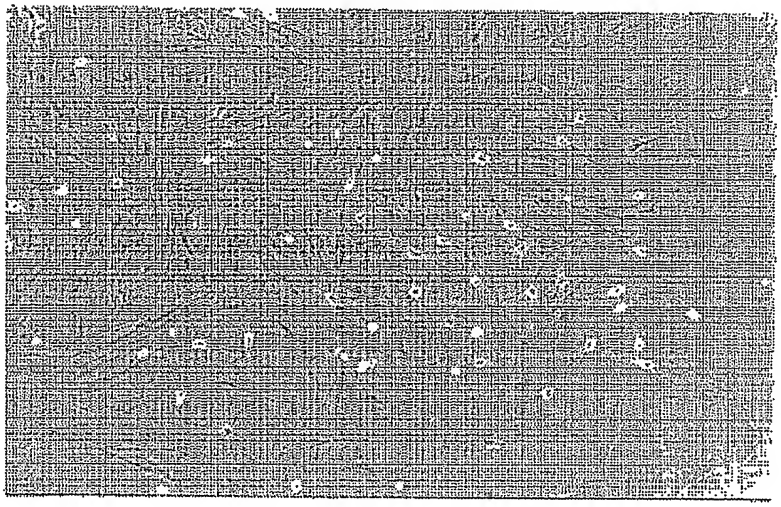

Fig. 11

photograph substituted
for drawing

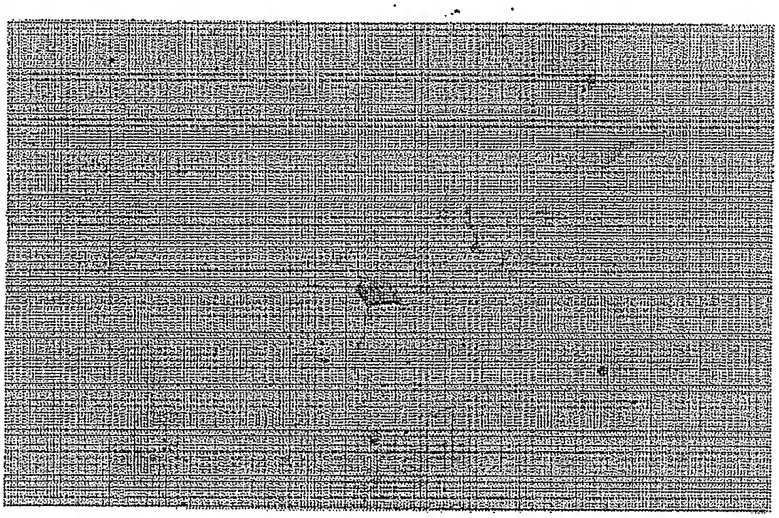

Fig. 12

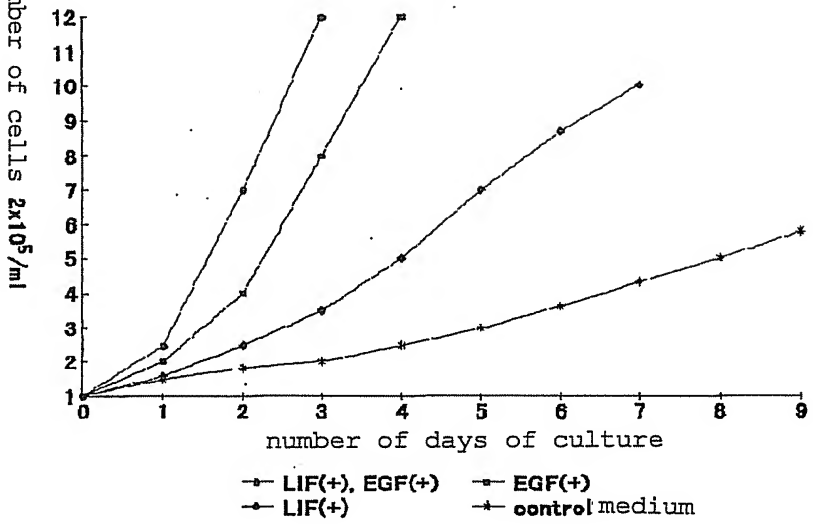

Fig. 13

photograph substituted for drawing